Eur J Clin Microbiol Infect Dis (2012) 31:1035–1039
DOI 10.1007/s10096-011-1402-z

ARTICLE

# Optimization of *Toxoplasma gondii* DNA extraction from amniotic fluid using NucliSENS easyMAG and comparison with QIAamp DNA minikit

**B. Faucher · F. Miermont · S. Ranque · J. Franck · R. Piarroux**

Received: 17 January 2011 / Accepted: 18 August 2011 / Published online: 4 September 2011


**Abstract** Antenatal diagnosis of congenital toxoplasmosis relies on PCR in amniotic fluid. Because parasitic load is often low, DNA extraction must be optimized. Manual methods remain widespread although automated methods appear more effective. This study aimed at optimizing an automated method and at comparing it with a widespread manual method: QIAamp DNA minikit. To optimize NucliSens easyMAG, we evaluated the addition of proteinase K pre-treatment and the increase of the amount of silica particles used for the extraction. The optimized method was then compared to QIAamp DNA minikit on samples containing less than 25 tachyzoites/ml. NucliSens easyMAG DNA yield was improved after proteinase K pre-treatment ($p<0.01$), but not with a higher silica particle input. The optimized method yielded more positive PCRs than the manual method, especially for samples containing 5 tachyzoites/ml or less (71% vs 26%, $p<10^{-4}$). The DNA amount in samples found positive by PCR was higher after optimized automated extraction than after manual extraction ($p<10^{-4}$). Proteinase K pre-treatment should be added to extract DNA from amniotic fluid using NucliSens easyMAG. Using this optimized automated method rather than manual methods would improve the sensitivity of *Toxoplasma* PCR and simplify the daily workflow.

B. Faucher (✉) · F. Miermont · S. Ranque · J. Franck · R. Piarroux
Laboratoire de Parasitologie-Mycologie,
Centre Hospitalo-Universitaire de la Timone,
264 rue St-Pierre,
13385 Marseille CEDEX 05, France
e-mail: benoit.faucher@ap-hm.fr

B. Faucher · R. Piarroux
Université de la Méditerrannée, UMR MD3,
Marseille, France

## Introduction

*Toxoplasma* PCR in amniotic fluid (AF) is the cornerstone of the antenatal diagnosis of congenital toxoplasmosis [1]. Highly sensitive methods are necessary because up to 46% of infected AF contain less than ten tachyzoites per milliliter (T/ml) [2]. However, accuracy varies because parasitic load is often low and sensitivity of extraction techniques varies [3]. An optimized DNA extraction is necessary to ensure an accurate PCR [4]. Currently, manual extraction methods are still widespread [5] though previous studies suggested that they might be inferior to automated methods [4]. The present study aimed at optimizing an automated method of DNA extraction (NucliSens easyMAG, bioMérieux) from AF, comparing this method with a widespread manual method (QIAamp DNA minikit, Qiagen). Because it was previously suggested that the automated extraction could benefit from the addition of proteinase K pre-treatment and a higher silica particle input [4], these two modifications of the extraction protocol were successively evaluated to define the optimized automated method.

## Materials and methods

### Sample preparation

The study design followed that of Yera et al. [4]. Briefly, *Toxoplasma gondii* tachyzoites (RH strain) were obtained from ascites of infected mice. They were purified by passaging the ascites fluid through 3-μm-pore-size polycarbonate membrane filters, numerated using Malassez cell, and diluted in phosphate-buffered saline (pH 7.4) to obtain suspensions of respectively 300, 750, 1500, 3000 and 7500 T/ml.

Negative AF samples were collected from patients experiencing seroconversion for *Toxoplasma,* in whom fetal contamination was excluded due to both a negative *Toxoplasma* PCR in AF and the disappearance of anti-*Toxoplasma* antibodies during the follow-up of the children. Three milliliters of these negative AF samples were taken. After osmotic lysis of erythrocytes and centrifugation at 1300 g for 10 minutes, they were resuspended as 190-µl aliquots using phosphate buffer saline. Ten microliters of phosphate buffer saline were added to two aliquots to obtain the control AF. Ten microliters of each calibrated parasitic suspension were then added to the remaining aliquots to obtain mimic AF samples containing respectively 1, 2.5, 5, 10 and 25 T/ml.

## DNA extraction

The automated DNA extraction was performed on the NucliSens easyMAG system following the manufacturer's Specific B protocol, with a 40-µl final elution volume. First, DNA was extracted using NucliSens easyMAG from 20 AF samples containing 25 T/ml, i.e. ten samples which had previously been treated with proteinase K for 30 min at 56°C and ten which had not. Second, DNA was extracted using NucliSens easyMAG from 30 additional samples, i.e. six with each concentration of *T. gondii* (1, 2.5, 5, 10 and 25 T/ml). These 30 samples were pre-treated with proteinase K. For each *Toxoplasma* concentration, 50 µl of the standard silica particle solution provided by the manufacturer were used for the DNA extraction of three AF samples and 100 µl were used for the other three samples. An additional comparison of the two amounts of silica particles was performed on 40 samples containing 1 T/ml. These results were used to set up the optimal extraction protocol using NucliSens easyMAG. Finally, this optimized method was compared to a manual DNA extraction method (QIAamp DNA minikit). Manual extraction included proteinase K pre-treatment for 2 h at 56°C. Final elution volume was 50 µl. Automated and manual DNA extraction were compared on 44 aliquots containing 1–25 T/mL and two control samples (Table 1).

## DNA amplification

*Toxoplasma* DNA was amplified by real-time PCR targeting the 529-bp repeat element as previously described [6], on the Roche LightCycler 480, using TaqMan probes. During the optimization study, two*Toxoplasma* PCRs were performed on each DNA extract. For the comparison of automated and manual methods, the number of *Toxoplasma* PCRs varied according to the parasitic concentration (Table 1). A DNA extract was considered positive if at least one *Toxoplasma* PCR was positive. Additionally, PCR inhibition was checked in each DNA extract by amplifying a noncompetitive internal control (mouse GALT gene), and human albumin gene was amplified to check that the extraction process had been successful. Comparison of *Toxoplasma* DNA yields between the two extraction methods were performed by comparing threshold cycles (Ct), which are the number of PCR cycles required to obtain enough *Toxoplasma* DNA to enable its detection. Ct levels are inversely proportional to the amount of target *Toxoplasma* DNA in the sample, i.e. each PCR cycle doubles the amount of target DNA.

**Table 1** Number of *Toxoplasma* PCR performed at different tachyzoite concentrations in the comparison study

| AF concentration (T/ml) | Number of DNA extractions | PCR per extract | Total PCR number |
|---|---|---|---|
| 0 | 1 | 6 | 6 |
| 1 | 5 | 6 | 30 |
| 2.5 | 5 | 6 | 30 |
| 5 | 5 | 3 | 15 |
| 10 | 5 | 3 | 15 |
| 25 | 2 | 3 | 6 |
| Total | 23 | | 102 |

*AF* amniotic fluid, *T/ml* tachyzoite of *Toxoplasma gondii* per ml

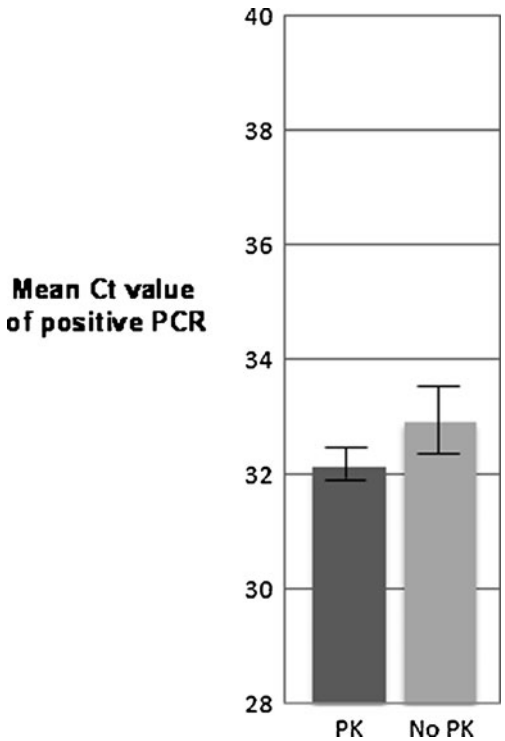


**Fig. 1** Cycle threshold (Ct) mean value of *Toxoplasma* PCR on amniotic fluids containing 25 tachyzoites/mL using NucliSens easyMAG with and without proteinase K pre-treatment

**Table 2** Comparison of optimized easyMAG and QIAamp DNA mini Kit performances

| AF load (T/ml) | NucliSENS easyMAG (bioMérieux) | | QIAamp DNA minikit (Qiagen) | |
|---|---|---|---|---|
| | Positive PCR | Positive extract[a] | Positive PCR | Positive extract[a] |
| 0 | 0/6 | 0/1 | 0/6 | 0/1 |
| 1 | 23/30 (77%) | 5/5 (100%) | 2/24 (8%) | 1/4 (25%)[b] |
| 2.5 | 15/30 (50%) | 3/5 (60%) | 8/30 (27%) | 4/5 (80%) |
| 5 | 15/15 (100%) | 5/5 (100%) | 8/15 (53%) | 5/5 (100%) |
| 10 | 15/15 (100%) | 5/5 (100%) | 10/15 (67%) | 4/5 (80%) |
| 25 | 6/6 (100%) | 2/2 (100%) | 5/6 (83%) | 2/2 (100%) |
| Infected AF (total) | 74/96 (77%) | 20/22 (91%) | 33/90 (37%) | 16/21 (76%) |

*AF* amniotic fluid, *T/ml* tachyzoite of *Toxoplasma gondii* per ml

[a] DNA extract considered positive if at least one *Toxoplasma* PCR detected parasitic DNA

[b] One extract was excluded from the analysis because human albumin gene was not amplified either

### Statistic analysis

The factors associated with a positive PCR result were analyzed using multivariate logistic regression. Those associated with the threshold amplification cycles (Ct) in positive PCRs were analyzed using multivariate analysis of variance with SAS® 9.1.3 (Cary, NC, USA). Generalized estimating equations were used to account for within replicates correlations.

## Results

### Proteinase K pre-treatment

The NucliSens easyMAG method exhibited a higher yield after pre-treatment with Proteinase K for 30 min at 56°C. All PCRs were positive in both groups but the Ct value was significantly lower for the PCRs performed on pre-treated samples (mean Ct value: 32.2 vs. 32.9, $p<0.01$, Fig. 1).

### Amount of silica

*Toxoplasma* PCRs yielded similar results with the two amounts of silica particles; 57% of PCRs (63% of extracts) were positive using 50 µl, compared to 54% of PCRs (60% of extracts) using 100 µl ($p=0.43$). For AF samples containing 1 T/ml, 43% of PCRs (48% of extracts) were positive using 50 µl of silica, compared to 37% of PCRs (43% of extracts) using 100 µl of silica ($p=0.57$). PCRs were always positive in both groups for samples containing more than 2.5 T/ml. In samples found positive by PCR, no difference of Ct value was found between the two groups ($p=0.57$).

### Comparison of NucliSens easyMAG and QIAamp DNA mini kit

The optimized automated method exhibited a higher yield in extracting *Toxoplasma* DNA than the manual method for all parasitic concentrations ($p<10^{-4}$). The difference was

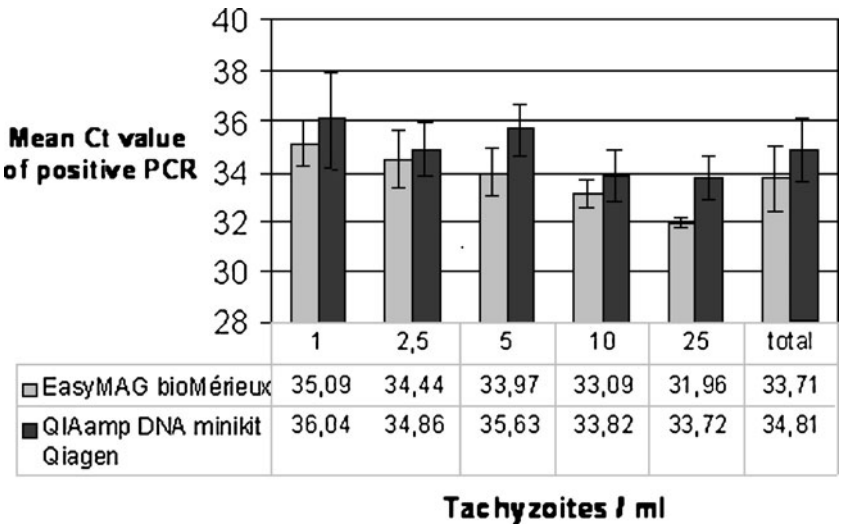


**Fig. 2** Cycle threshold (Ct) mean value of *Toxoplasma* PCR on amniotic fluid using optimized NucliSens easyMAG and QIAamp DNA mini Kit

highest for samples containing 1 T/ml (Table 2). After automated extraction, the Ct value of a positive PCR was significantly lower, whatever the concentration of tachyzoites (33.7 vs 34.8 on average, $p<10^{-4}$) (Fig. 2). Because several PCRs were performed on each DNA extract, the two methods yielded similar results in identifying positive extracts; 87% and 71% of samples containing 5 T/mL or less were found positive after automated and manual extraction, respectively. No PCR inhibitor was detected. Human albumin gene could not be amplified in one DNA extract obtained with the manual method, which was excluded from subsequent analysis.

## Discussion

Proteinase K pre-treatment, which helps with lysing parasite cell walls, induced a mild but significant increase of the amount of *Toxoplasma* DNA obtained with NucliSens easyMAG; the observed decrease of 0.7 Ct suggests that 1.6 times more *Toxoplasma* DNA were yielded. Though mild, this difference might be important in the diagnosis of congenital toxoplasmosis as the parasitic load is often very low in infected AF [2]. Conversely, increasing the input of silica particles did not improve the extraction yield of NucliSens easyMAG. This result refutes the hypothesis that the capacity of silica particles to bind DNA, a necessary step of the extraction process, was exceeded [4]. However, this result should not be extrapolated to other biologic fluids that might contain more cells. Eventually, the optimized NucliSens easyMAG method only included the addition of proteinase K pre-treatment.

The optimized automated method exhibited a higher yield in extracting *Toxoplasma* DNA than the manual method for all concentrations of tachyzoites. The difference was highest for low parasitic loads. A previous study similarly reported that the QIAamp DNA minikit exhibited a lower yield in extracting *Toxoplasma* DNA than two automated methods, namely, MagNA Pure Compact (Roche) and BioRobot EZ1 (Qiagen), but not lower than NucliSens easyMAG [4]. It was then hypothesized that the absence of proteinase K pre-treatment explained the difference between NucliSens easyMAG and the two other automated methods. Our results support this hypothesis.

QIAamp DNA minikit yielded less positive results in the present study than in a previous report [4]. However, our results are similar to most previous reports, i.e. 39.5% of the samples containing 5 T/ml were misdiagnosed in an international study [7], which was confirmed in a recent multicenter study [8]. In the present study, no methodological bias can explain the differences between the two methods, i.e. samples were randomly allocated to undergo manual or automated extraction, and the analyses were performed on the same days by the same operator who routinely used both methods. Our results are supported by earlier reports which showed that manual extraction methods were comparable [9–11] or less sensitive [12–15] than automated methods for other types of samples and micro-organisms. Besides, automated methods are associated with additional benefits, e.g. they are time-saving, they decrease the risk of human error, and they enable sample tracing, bar code reading, and reagent logging.

Interestingly, no PCR inhibitor was detected in the present study, although the mimic AF samples were obtained from routine AF and not from hydramnios. Conversely, presence of PCR inhibitors in AF was already reported in previous studies with other extraction methods [4].

Finally, performing several *Toxoplasma* PCRs on each AF sample increased the chance of detecting the parasite. This strategy was recommended previously [5] because the sensitivity of *Toxoplasma* PCR decreases when parasitic load is below 10 T/ml [3, 7, 16]. This strategy indeed enabled correction of more than half of initial false negative results in the present study.

## Conclusion

Proteinase K pre-treatment improved the yield of *Toxoplasma* DNA extraction from AF using the NucliSens easyMAG method. This optimized automated method appeared more sensitive than a widespread manual method, namely, QIAamp DNA minikit, especially for low parasitic loads. These results support the use of automated methods for the antenatal diagnosis of congenital toxoplasmosis.

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

1. Filisetti D, Gorcii M, Pernot-Marino E, Villard O, Candolfi E (2003) Diagnosis of congenital toxoplasmosis: comparison of targets for detection of *Toxoplasma gondii* by PCR. J Clin Microbiol 41:4826–4828
2. Costa JM, Ernault P, Gautier E, Bretagne S (2001) Prenatal diagnosis of congenital toxoplasmosis by duplex real time PCR using fluorescence resonance energy transfer hybridization probes. Prenat Diagn 21:85–88
3. Bastien P, Jumas-Bilak E, Varlet-Marie E, Marty P, The ANOFEL Toxoplasma-PCR Quality Control Group (2007) Three years of multi-laboratory external quality control for the molecular detection of *Toxoplasma gondii* in amniotic fluid in France. Clin Microbiol Infect 13:430–433
4. Yera H, Filisetti D, Bastien P, Ancelle T, Thulliez P, Delhaes L (2009) Multicenter comparative evaluation of five commercial methods for *Toxoplasma* DNA extraction from amniotic fluid. J Clin Microbiol 47:3881–3886

5. Sterkers Y, Varlet-Marie E, Marty P, Bastien P, The ANOFEL Toxoplasma-PCR Quality Control Group (2010) Diversity and evolution of methods and practices for the molecular diagnosis of congenital toxoplasmosis in France: a four years survey. Clin Microbiol Infect 16:1594–1602
6. Homan WL, Vercammen M, De Braekeleer J, Verschueren H (2000) Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in *Toxoplasma gondii*, and its use for diagnostic and quantitative PCR. Int J Parasitol 30:69–75
7. Kaiser K, Van Loon AM, Pelloux H, Ferrandiz J, Picot S, Wallace P, Peyron F (2007) Multicenter proficiency study for detection of *Toxoplasma gondii* in amniotic fluid by nucleic acid amplification methods. Clin Chim Acta 375(1–2):99–103
8. Sterkers Y, Varlet-Marie E, Cassaing S, Brenier-Pinchart MP, Brun S, Dalle F, Delhaes L, Filisetti D, Pelloux H, Yera H, Bastien P (2010) Multicentric comparative analytical performance study for molecular detection of low amounts of *Toxoplasma gondii* from simulated specimens. J Clin Microbiol 48(9):3216–3222
9. Chan KH, Yam WC, Pang CM, Chan KM, Lam SY, Lo KF, Poon LL, Peiris MJ (2008) Comparison of the NucliSens easyMAG and Qiagen BioRobot 9604 nucleic acid extraction systems for diagnosis of RNA and DNA respiratory viruses on nasopharyngeal aspirates. J Clin Microbiol 46:2195–2199
10. Perandin F, Pollara PC, Gargiulo F, Bonfanti C, Manca N (2009) Performance evaluation of the automated NucliSens easyMAG nucleic acid extraction platform in comparison with QIAamp Mini kit from clinical specimens. Diagn Microbiol Infect Dis 64 (2):158–165
11. Huijsmans C, Damen J, Van Der Linden J, Savelkoul P, Hermans M (2010) Comparative analysis of four methods to extract DNA from paraffin-embedded tissues: effect on downstream molecular applications. BMC Res Notes 14(3):239
12. Dundas N, Leos NK, Mitui M, Revell P, Rogers BB (2008) Comparison of automated nucleic acid extraction methods with manual extraction. J Mol Diagn 10(4):311–316
13. Francesconi A, Kasai M, Harrington SM, Beveridge MG, Petraitiene R, Petraitis V, Schaufele RL, Walsh TJ (2008) Automated and manual methods of DNA extraction for *Aspergillus fumigatus* and *Rhizopus oryzae* analyzed by quantitative real-time PCR. J Clin Microbiol 46(6):1978–1984
14. Loens K, Bergs K, Ursi D, Goossens H, Ieven M (2007) Evaluation of NucliSens easyMAG for automated nucleic acid extraction from various clinical specimens. J Clin Microbiol 45 (2):421–425
15. Pillet S, Bourlet T, Pozzetto B (2009) Comparative evaluation of a commercially available automated system for extraction of viral DNA from whole blood: application to monitoring of Epstein-Barr virus and cytomegalovirus load. J Clin Microbiol 47(11):3753–3755
16. Edvinsson B, Jalal S, Nord CE, Pedersen BS, Evengard B, The ECSMID Study Group on Toxoplasmosis (2004) DNA extraction and PCR assays for detection of *Toxoplasma gondii*. APMIS 112:342–348